Amaranthus spinosus, Leonotis nepetaefolia, and *Leonurus sibiricus*: New Hosts of *Phomopsis* spp. in Brazil

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ABSTRACT

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Phomopsis spp. was isolated from *Amaranthus spinosus, Leonotis nepetaefolia,* and *Leonurus sibiricus,* common weeds in soybeans grown in southern Brazil. The latter two hosts were symptomless carriers of *Phomopsis* spp. Pycnidia produced by cultures of *Phomopsis* spp. isolated from the three weed hosts gave rise to alpha and beta conidia typical of *Diaporthe phaseolorum.* The *Phomopsis* spp. isolates from *L. nepetaefolia* and *L. sibiricus,* but not from *A. spinosus,* reduced seed germination, radicle length, and emergence of UFV₁ soybeans but not of Rico 23 common bean (*Phaseolus vulgaris*) grown in infested sand and soil. These weed isolates colonized soybean stems and produced pycnidia. Isolates from *A. spinosus* did not produce pycnidia on common bean stems.

Pod and stem blight, stem canker, and Phomopsis seed decay of soybean (*Glycine max* (L.) Merr.), caused by *Phomopsis* spp. and its anamorphs, *Diaporthe phaseolorum* (Cke. & Ell.)

Sacc. var. *sojae* Wehm. and *D. phaseolorum* (Cke. & Ell.) Sacc. var. *caulivora* Athow & Caldwell, are worldwide in distribution (11,17). Previous workers equated symptom development with parasitism by *Phomopsis* spp. (2,9,13,15); however, *Phomopsis* spp. can colonize immature soybean plants without symptom production (5,11,12). Therefore, host range studies that rely only on symptoms may underestimate the host range and parasitic ability of this fungus.

Weeds serve as alternative hosts of pathogens that affect soybean seeds (4,8,18). A correlation between the occurrence of *Colletotrichum dematium, Phomopsis* spp., and *Fusarium semitectum* on weed hosts in the field with seed infection by these fungi was reported in Brazil (7).

We wanted to determine if infection of weeds by *Phomopsis* spp. was important in tropical soybean-growing areas, where weeds pose a greater problem than in temperate regions (6). Results from a portion of this study have been published (4).

MATERIALS AND METHODS

All the studies were conducted at the Universidade Federal de Viçosa, Viçosa, Brazil (UFV).

Isolation of *Phomopsis* spp. Isolations were made from green plants with mature seeds of *Amaranthus spinosus* L., *Leonotis nepetaefolia* (L.) R. Br., and *Leonurus sibiricus* L., common weeds found in soybean fields in southern Brazil. Stem sections were cut from five plants each of *L. nepetaefolia* and *L. sibiricus* collected from the UFV Experimental Farm at Viçosa. Stems from five plants of *A. spinosus* were collected at the UFV Experimental Farm, and the Florestal Agricultural School farm 290 km from Viçosa. Stems of each plant species were kept separate, cut into pieces 3–5 mm long, and surface-disinfested as described previously (5). The stem pieces were plated on potato-dextrose agar (PDA) amended with 100 µg/ml streptomycin sulfate in 9-cm-diameter culture plates. Isolations were made from margins of lesions in *A. spinosus* stems with symptoms of dieback. The tissues were treated as described previously and plated on PDA with streptomycin sulfate. Stems of *L. sibiricus* plants inoculated with an isolate of *Phomopsis* sp. from symptomless plants of the same species were surface-disinfested and immersed in 11.6%

paraquat as described previously (5). Two hundred seeds from each weed host were also surface-disinfested as described previously and assayed on PDA.

Inoculum preparation. The isolates of *Phomopsis* spp. recovered from stems of *A. spinosus, G. max, L. nepetaefolia,* and *L. sibiricus* were cultured separately in 125-ml culture flasks containing soybean seed-extract broth for 14 days at 25 C. The broth was prepared by boiling 1 L of water containing 100 g of soybean seeds for 10 min. The extract was filtered through cotton gauze and the filtrate volume adjusted to 1 L with distilled water, then 20 g of sucrose was added and the material autoclaved (121 C for 15 min).

After 14 days of fungal growth, the broth was decanted and the mycelial mats triturated in a sterile blender at high speed for 1 min with 100 ml of sterile distilled water.

Pathogenicity studies. Seeds of UFV₁ soybean and Rico 23 common bean (*Phaseolus vulgaris* L.) were surface-disinfested as described previously for weed stem pieces and placed on moist paper towels in 15-cm-diameter culture plates. Each plate contained 40 seeds, with four replicates (plates) per isolate. Each seed was treated with about 1 ml of one of the mycelial suspensions. Surface-disinfested seeds treated with 1 ml of sterile distilled water served as the control. Seeds were incubated in a chamber programmed for alternate 12-hr light and dark at 22–24 C under six white fluorescent tubes (30 μEinsteins^{-2}S^{-1}). Percentage germination and radicle length were recorded after 7 days for UFV₁ and after 4 days for Rico 23. A seed was considered germinated if the radicle length was greater than 2.5 cm.

Pathogenicity of the isolates also was studied using either soil or sand treated earlier with methyl bromide. UFV₁ soybean and Rico 23 bean seeds were surface-disinfested and inoculated as described previously and placed in plastic trays containing either sand or soil (3:1, field soil/aged manure). For each isolate of *Phomopsis* sp., three replicates of 40 seeds each were planted. Emergence counts were recorded after 10 days. Plants were rated visually after 30 days for *Phomopsis* spp. pycnidia.

Inocula of the two isolates of *Phomopsis* spp. from *L. sibiricus* were prepared by flooding 22-day-old PDA culture plates with 25 ml of sterile distilled water, scraping the surface of the culture to remove the mycelium, and macerating the suspension in a sterile mortar and pestle. UFV₁ seeds were surface-disinfested, inoculated with the mycelial suspension as described previously, and tested separately on blotter paper and in sand and soil. The same procedure was repeated except seeds were placed in a mycelial suspension of each isolate under vacuum (1 bar) for 2 min before

being tested on blotter paper and in sand or soil.

In addition, pathogenicity of the two isolates of *Phomopsis* spp. from *L. sibiricus* was tested using 38-day-old UFV₁ plants grown in 20-cm-diameter greenhouse pots containing soil treated with methyl bromide. There were four plants in each of two pots for each treatment. A sterile scalpel was used to make a slit about 0.5 cm in the stem at about one-third the height of the plant. A small portion of mycelium from an 18-day-old PDA culture of one of the isolates was placed in the wound and the inoculated area was covered with Parafilm. Stems with sterile blocks of PDA placed in the slits served as a control. After 39 days in the greenhouse, stem pieces 5 mm long were cut 2 cm below the inoculation point. They were surface-disinfested and placed on PDA with streptomycin sulfate as described previously.

Inoculation of weed hosts. Seeds from each of the three weed hosts were germinated on moist blotter paper, transplanted into vermiculite, then transplanted again after 3 wk into 10-cm-diameter pots containing pasteurized soil. One to three pots with two plants per pot were prepared. Each weed plant was sprayed with a mycelial suspension of an isolate of *Phomopsis* sp. from the respective weed host. The mycelial suspension was prepared from cultures grown in soybean seed-extract broth. Plants sprayed with water served as a control. All plants were maintained in a growth chamber programmed for alternate 12-hr light and dark, relative humidity approaching 100%, and 22–24 C. After 72 hr, all plants were transferred to a greenhouse bench and observed for symptom development.

Means for germination, emergence, and radicle length were compared using Duncan's multiple range test for significance.

RESULTS

On PDA, the mycelium of *Phomopsis* spp. from *L. sibiricus* and *L. nepetaefolia* was identical in appearance to isolates obtained from soybean. Mycelium of *Phomopsis* spp. isolates from *A. spinosus* generally was darker brown than the soybean isolates and had yellowish borders. Alpha conidia were one-celled, hyaline, cylindrical to elliptical with rounded ends, and varied 7–8 × 2–4 μm (average 7.8 × 2.8 μm) from *A. spinosus*, 5.8–7.6 × 2.3–3.5 μm (average 6.6 × 2.8 μm) from *L. sibiricus*, and 4.3–5.4 × 1.4–2.3 μm (average 4.8 × 1.9 μm) from *L. nepetaefolia*. Beta conidia were one-celled, hyaline, often with one end of the conidium curved and tips of conidia rounded, and varied 18–26 × 1–2 μm (average 21 × 1.5 μm) from *A. spinosus*, 15.2–21.1 × 12 μm (average 18.4 × 12 μm) from *L. sibiricus*, and 15.1–24.8 × 1.1–1.9 μm (average 20.2 × 1.5 μm) from *L. nepetaefolia*.

Phomopsis spp. was not isolated from seeds of *A. spinosus* or *L. sibiricus* and was only isolated from 1% of seeds of *L. nepetaefolia*.

Plants of *L. nepetaefolia* and *L. sibiricus* inoculated with their respective isolates of *Phomopsis* spp. did not show symptoms until plant maturity and senescence. Pycnidia were sparse and first became evident at 10 and 14 days before plants were dry (dead). In contrast, infected plants of *A. spinosus* in the field showed tip and stem dieback at intermediate and later plant growth stages. Pycnidia developed on young or old stem pieces. Pycnidia on stems from

Table 1. Pathogenicity of *Phomopsis* spp. isolates from soybean and *Leonorus sibiricus* on cultivar UFV₁ soybean seeds

	Seed test		Emergence	
Isolate	**Germination**[w] **(%)**	**Radicle length**[x] **(mm)**	**Sand**[y] **(%)**	**Soil**[y] **(%)**
	Test 1			
Control	75.2 a[z]	59.4 a	74.2 a	68.7 ab
Soybean isolate 1	62.2 b	55.8 a	74.2 a	64.4 bc
Soybean isolate 2	60.5 b	54.8 a	68.6 abc	63.1 c
L. sibiricus isolate 1	48.3 c	48.1 ab	73.3 ab	61.0 c
	Test 2			
Control	69.5 a	66.9 a	53.4 ab	57.9 a
L. sibiricus isolate 1	⋯	⋯	47.4 ab	43.5 b
L. sibiricus isolate a	22.2 b	18.2 b	42.6 b	27.7 c

[w] Arc sine transformed percentage germination of seeds surface-disinfested in 95% EtOH for 5 sec followed by 0.5% NaOCl for 4 min. Means of four replicates of 40 seeds each; ⋯ = data not available. Plates were incubated with 12 hr light and for 7 days at 22–24 C.

[x] A seed was considered germinated if the radicle length was greater than 2.5 cm; ⋯ = data not available.

[y] Arc sine transformed percentage emergence in sterile sand and soil evaluated after 10 days; means of three replicates of 40 seeds each.

[z] Means within each test followed by the same letter are not significantly different according to Duncan's multiple range test at $P = 0.05$.

Table 2. Pathogenicity of *Phomopsis* spp. isolates from three weed hosts and from *Glycine max* on seed and seedlings of cultivars UFV₁ soybean and Rico 23 common bean

	UFV₁				Rico 23			
	Seed test		Emergence		Seed test		Emergence	
Isolate source	Germination[w] (%)	Radicle length[x] (mm)	Sand[y] (%)	Soil (%)	Germination[w] (%)	Radicle length[x] (mm)	Sand[y] (%)	Soil (%)
Leonurus sibiricus	45.0 b[z]	54.2 bcd	66.2 bc	58.2 bc	50.1 a	44.6 a	79.4 abc	67.6 abc
Leonotis nepetaefolia	46.9 b	65.8 bc	51.3 c	65.4 abc	58.0 a	54.8 a	75.0 abc	85.7 a
Amaranthus spinosus	68.7 a	95.1 a	69.7 ab	60.6 abc	56.5 a	54.0 a	75.1 abc	84.7 a
Glycine max	21.8 c	34.2 d	55.4 bc	57.3 bc	52.3 a	45.3 a	80.4 ab	61.2 c
Control	66.7 a	76.4 ab	75.6 a	60.5 abc	51.7 a	47.5 a	65.6 bc	80.1 ab

[w] Arc sine transformed percentage germination of seeds surface-disinfested in 95% EtOH for 5 sec followed by 0.5% NaOCl for 4 min. Means of four replicates of 40 seeds each. Plates incubated with 12 hr light at 22–24 C. Germination of soybean seeds read at 7 days and beans at 4 days after planting.
[x] A seed was considered germinated if the radicle length was greater than 2.5 cm.
[y] Arc sine transformed percentage emergence in sterile sand and soil evaluated after 10 days; means of three replicates of 40 seeds each.
[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test at $P = 0.05$.

A. spinosus varied in size: 162–464 × 93–267 μm (average 247 × 170 μm), 221–465 × 186–407 μm (average 384 × 350 μm) on *L. sibiricus*, and 114–227 × 109–175 μm (average 160 × 139 μm) on *L. nepetaefolia*.

The *Phomopsis* sp. isolate from *L. sibiricus* significantly ($P = 0.05$) reduced seed germination compared with the control and the two soybean isolates in test 1 (Table 1). Radicle lengths and emergence from sand and soil were similar to the control. Emergence in sand was greater than in soil in most cases.

In a second test using another isolate from *L. sibiricus*, seed germination and radicle lengths were significantly ($P = 0.05$) reduced compared with the control (Table 1). Seed germination in soil after treatment with this second isolate was significantly lower than that of the first or the control isolate but similar in sand. Vacuum-infiltration of seeds with the mycelial suspension did not alter pathogenicity results. UFV₁ seeds treated with a *Phomopsis* sp. isolate from *A. spinosus* had similar germination, radicle length, and emergence from soil to uninoculated seeds (Table 2). Uninoculated seeds showed the highest germination and emergence from sand, whereas emergence from soil and radicle length were intermediate. Germination and radicle length of seeds treated with isolates from *L. sibiricus* and *L. nepetaefolia* were significantly ($P = 0.05$) less than for uninoculated seeds or seeds inoculated with the *A. spinosus* isolate but greater than for seeds treated with the soybean isolate. Lowest emergence from sand was observed with the *L. nepetaefolia* isolate, whereas in soil, emergence was greater (but not significantly) than from uninoculated seeds. Seeds treated with the *L. sibiricus* isolate had intermediate levels of emergence in sand and soil. After 1 mo, soybeans previously inoculated with *Phomopsis* spp. weed isolates showed pycnidia in stems.

No significant differences in seed germination, radicle length, or emergence in sand were observed when Rico 23 seeds were treated with the *Phomopsis* spp. isolates from any weed host compared with uninoculated control seed or seed inoculated with the soybean isolate. Emergence in soil was lowest in seed treated with the *Phomopsis* sp. isolate from soybean. After 1 mo, *P. vulgaris* plants inoculated with *Phomopsis* spp. isolates from soybean, *L. sibiricus*, and *L. nepetaefolia*, but not from *A. spinosus*, showed pycnidia on stems after incubation in a moist chamber.

DISCUSSION

Mycelial characteristics of *Phomopsis* spp. weed isolates on PDA were similar to those described for soybean isolates (17), and mean pycnidial and conidial measurements of *Phomopsis* spp. from *A. spinosus*, *L. nepetaefolia*, and *L. sibiricus* were within the range for those from *D. phaseolorum* var. *sojae* (15,17). This is the first report of *Phomopsis* spp. isolated from *L. sibiricus*, *L. nepetaefolia*, and *A. spinosus*. The first two weeds are commonly found in soybean fields in southern Brazil during summer, fall, and after soybean harvest. *A. spinosus* is found in soybean fields during the growing season (14).

Isolation of *Phomopsis* spp. from apparently healthy tissue, lack of symptom development during the growing season, and rapid pycnidial development before host desiccation indicates latent colonization of *L. nepetaefolia* and *L. sibiricus* by *Phomopsis* spp. Pycnidial development on stems was less extensive, however, than that observed on soybeans (11,12). Latent infection of weeds by *Phomopsis* spp. has also been reported on *Abutilon theophrasti*, and the *Phomopsis* spp. isolates were pathogenic to soybean (8). Symptomless infection of noncrop hosts by fungi pathogenic to important crops has been observed previously (10,16,19).

Weeds can serve as alternative hosts for pathogens that affect soybean seeds (8), and correlations between weed infestation and soybean seed quality have been noted (1,7). Our *Phomopsis* spp. isolates from *L. sibiricus* and *L. nepetaefolia* were pathogenic to soybean seeds and plants. Isolates from *A. spinosus* did not affect seed germination although stems were subsequently colonized with pycnidia. All *Phomopsis* spp. weed isolates were nonpathogenic on seeds of *P. vulgaris* Rico 23 (3), however, although the *Phomopsis* spp. isolates from *G. max*, *L. sibiricus*, and *L. nepetaefolia* produced pycnidia on stems of this cultivar.

A. spinosus, *L. nepetaefolia*, and *L. sibiricus* serve as alternative hosts for *Phomopsis* spp., and because they often occur during and after soybean harvest, they permit *Phomopsis* spp. to remain active between growing seasons. In tropical regions, overseasoning of *Phomopsis* spp. on weed hosts may increase inoculum, providing for increased seed infection and decreased seed quality in the subsequent season. In addition to management of crop residue (12), weed control after soybean harvest may be necessary to reduce *Phomopsis* spp. inoculum levels.

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